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Coordinated Action of CK1 Isoforms in Canonical Wnt Signaling[▽]

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Activation of the Wnt pathway promotes the progressive phosphorylation of coreceptor LRP5/6 (low-density lipoprotein receptor-related proteins 5 and 6), creating a phosphorylated motif that inhibits glycogen synthase kinase 3β (GSK-3β), which in turn stabilizes β-catenin, increasing the transcription of β-catenin target genes. Casein kinase 1 (CK1) kinase family members play a complex role in this pathway, either as inhibitors or as activators. In this report, we have dissected the roles of CK1 isoforms in the early steps of Wnt signaling. CK1ε is constitutively bound to LRP5/6 through its interaction with p120-catenin and E-cadherin or N-cadherin and is activated upon Wnt3a stimulation. CK1α also associates with the LRP5/6/p120-catenin complex but, differently from CK1ε, only after Wnt3a addition. Binding of CK1α is dependent on CK1ε and occurs in a complex with axin. The two protein kinases function sequentially: whereas CK1ε is required for early responses to Wnt3a stimulation, such as recruitment of Dishevelled 2 (Dvl-2), CK1α participates in the release of p120-catenin from the complex, which activates p120-catenin for further actions on this pathway. Another CK1, CK1γ, acts at an intermediate level, since it is not necessary for Dvl-2 recruitment but for LRP5/6 phosphorylation at Thr1479 and axin binding. Therefore, our results indicate that CK1 isoforms work coordinately to promote the full response to Wnt stimulus.

The Wnt pathway plays diverse roles in embryonic development and has been implicated in human diseases, including cancer (9). A key element in this pathway is the E-cadherin-associated protein β-catenin. When released from the junctional complex, β-catenin translocates to the nucleus, where it interacts with the Tcf family of transcriptional factors and regulates the expression of a variety of genes. The translocation of β-catenin to the nucleus is tightly controlled by the activity of a complex involved in β-catenin degradation. This complex includes the product of the tumor suppressor adenomatous polyposis coli (APC) gene, axin, and the associated Thr/Ser protein kinases, CK1α and glycogen synthase kinase 3β (GSK-3β) (12). As a result of the activity of this complex, β-catenin is phosphorylated and degraded by the proteasome. The activity of the degradation complex is blocked by canonical Wnt factors, which activate a signaling pathway leading to the stabilization of cytosolic β-catenin (12, 13).

Wnt ligands form a complex with low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6) and Frizzled (Fz) receptors (7). Upon Wnt ligand binding, the LRP5/6 cytosolic domain gets phosphorylated in different residues by the action of several protein kinases (15). For, instance Thr1479 is phosphorylated by casein kinase 1γ (CK1γ), a modification that is also dependent on the Fz-associated protein Dishevelled (Dvl) (1, 4, 28). Other members of this family, such as CK1ε and CK1α, also contribute to the phosphorylation of LRP5/6 and Dvl (17, 21, 27). Phosphorylation of LRP5/6 promotes the recruitment of axin and GSK-3β to the complex (14, 27); moreover, it creates binding sites for GSK-3β in PPPSPxS motifs located in the C-terminal domain that inhibit this enzyme (16, 24). Recent results indicate that the GSK-3β bound to the LRP5/6 complex is internalized in multivescular endosomes sequestering GSK-3β from its cytosolic targets (22). As a consequence, β-catenin phosphorylation by GSK-3β is prevented, and β-catenin half-life is increased.

We have described how CK1ε is constitutively bound to p120-catenin. Interaction with this protein is required for the association of CK1ε to LRP5/6 (2). CK1ε/p120-catenin does not bind directly to LRP5/6 but through the interaction of both proteins to E-cadherin (2). The p120-catenin–CK1ε interaction is functionally relevant since depletion of p120-catenin prevents CK1ε activation upon Wnt3a stimulation and early responses to Wn3a, such as LRP5/6 and Dvl-2 phosphorylation and axin recruitment to the signalosome (2). Moreover, in response to Wnt3a, p120-catenin is phosphorylated at Ser268, a modification dependent on CK1ε activity, which disrupts its interaction with E-cadherin and, subsequently, with LRP5/6, promoting the release of CK1ε/p120-catenin from the Wnt receptor complex (2). E-cadherin-unbound p120-catenin plays additional roles in Wnt signaling since it controls the transcriptional activity of Kaiso transcriptional factor (5). In this article, we study the association of the different CK1 isoforms with the complex and dissect their different contributions to the early events triggered by Wnt signaling.

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MATERIALS AND METHODS

Cell culture. SW-480-ADH epithelial cells established from a primary colon adenocarcinoma were used in these experiments. Although these tumoral cells contain a mutated form of APC and stabilized β-catenin, the initial responses to Wnt ligands are identical to other cell lines previously used to study this pathway (2). Alternatively, HEK293 cells or murine embryonic fibroblasts (MEFs) were used. Assays were performed in these cells when they were 60 to 80% confluent. Control L fibroblasts or fibroblasts stably transfected with a plasmid encoding Wnt3a were obtained from the ATCC and were cultured in medium containing 0.4 mg/ml G-418. When indicated, cells were treated with conditional medium from control or Wnt3a-expressing cells for the indicated times.

Cell transfection and selection of transfectants. Human short hairpin RNAs (shRNAs) specific for p120-catenin, CK1α, CK1ε, CK1γ, and axin 1 and irrelevant control shRNA (clone SCH002) were all obtained from Sigma. We selected those with the greatest effects on the expression of the corresponding protein, which were TRCN0000039666, TRCN0000006042, TRCN0000001837, TRCN0000038675, and TRCN0000061799, for p120-catenin, CK1α, CK1ε, CK1γ, and axin 1, respectively. For stable expression of p120-catenin shRNA, SW-480 or HEK293 cells were infected with lentiviral particles containing an shRNA targeting p120-catenin. Infected cells were selected with puromycin at 1 μg/ml. Control cells were infected with lentivirus bearing a nontargeting shRNA (clone SCH002; Sigma). Stable cell populations of human CK1α, CK1ε, CK1γ, or axin 1 shRNAs were generated by transfecting SW-480 or HEK293 cells with specific shRNA using Lipofectamine 2000 (Invitrogen) as described by the manufacturer. After incubation with the reaction mixture for 5 to 6 h, cells were washed twice with Opti-MEM without antibiotics and then cultured in Dulbecco's modified Eagle's medium (DMEM)–10% fetal calf serum (FCS) for 72 h before selection with 2 μg/ml puromycin.

Preparation of DNA constructs, purification of recombinant proteins, and pulldown assays. The generation of the bacterial expression plasmid pGEX-6P encoding the glutathione *S*-transferase (GST) protein fused to wild-type p120-catenin isoform 1, encompassing amino acids (aa) 1 to 911, and isoform 3, encompassing amino acids (aa) 102 to 911, has been previously reported (2). The generation of the expression plasmid pGEX encoding GST fused to the cytosolic domain of N-cadherin (GST-cytoN-cadh; aa 746 to 906) or E-cadherin (GST-cytoE-cadh; aa 732 to 883) has been described previously (18). GST fusion proteins were expressed in *Escherichia coli* and purified by affinity chromatography on glutathione-Sepharose as described previously (3). When required, GST was removed by cleaving with Pre-Scission protease (GE Healthcare). Where indicated, GST proteins (10 pmol) were phosphorylated with 300 mU of recombinant protein kinase CK1 (aa 1 to 237 of CK1δ, corresponding to the catalytic domain common to all the CK1 isoforms; Sigma), in a final volume of 50 μl in the following conditions: 9 mM $MgCl_2$,0.5 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM EDTA, 2.5 mM β-glycerol phosphate (pH 7.0), and 0.1 mM ATP. Reactions were performed for 40 min at 30°C. Pulldown assays were performed using purified recombinant proteins fused to GST as bait and SW-480 or HEK293 cell extracts. Glutathione-Sepharose-bound proteins were analyzed by Western blotting with specific monoclonal antibodies against p120-catenin, E-cadherin, N-cadherin, or LRP5/6 (see below). The polyclonal antibody to the GST protein was from GE Healthcare (reference no. 27-4577-01). Immunoblots were analyzed with the SNAP protein detection system (Millipore). All binding assays were repeated at least three times.

Immunoprecipitation assays. Cell extracts were prepared by homogenizing cells in 1% digitonin lysis buffer (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1 mM EDTA, 1% digitonin), supplemented with protease inhibitors (0.3 μM aprotinin, 1 μM leupeptin, 1 μM pepstatin, 1 mM Pefabloc) and phosphatase inhibitors (10 mM NaF, 0.1 mM sodium orthovanadate, and 2.5 mM β-glycerol phosphate). After passing cells 10 times through a 20-gauge syringe, extracts were left on ice for 10 min and centrifuged at 14,000 × *g* for 5 min at 4°C. The supernatants constituted the cell extracts. Proteins were immunoprecipitated from cell extracts (300 to 600 μg), using 2 μg/ml of the appropriate antibody or an irrelevant IgG as a control, for 16 h at 4°C. Samples were incubated with 30 μl of γ-bind G-Sepharose (GE Healthcare) for an additional 2 h at 4°C. Immunoprecipitates were washed three times with phosphate-buffered saline (PBS)–0.1% NP-40, and bound proteins were eluted with electrophoresis sample buffer. Immunoprecipitated proteins were analyzed by Western blotting using antibodies specific for p120-catenin, E-cadherin, N-cadherin, and CK1ε (all monoclonal antibodies [MAbs] from BD Biosciences; references 610134, 610182, and 610920, and 610445, respectively); axin 1, CK1α, Fz, and Dvl-2 (Santa Cruz Biotechnology; reference no. sc-14029, sc-6477, sc-7429, and sc-13974, respectively); CK1γ (Abcam; reference no. ab64829); LRP5/6 (Acris; reference no. Ap00345PU-N); phospho Thr1479 LRP5/6 (Abnova; reference no. PAB12632); or phospho Ser268 in p120-catenin (kindly provided by A. B. Reynolds [25]).

Analysis of protein distribution by immunofluorescence. SW-480 cells were cultured on coverslips after depletion of the indicated proteins as described above. Cells were treated with control or Wnt3a medium for 30 min, rinsed three times with PBS, fixed with 4% paraformaldehyde for 15 min, washed three times with PBS, permeabilized with PBS–0.2% Triton X-100 for 5 min, and washed exhaustively with PBS. Cells were blocked with 3% bovine serum albumin in PBS for 30 min at 20°C. A 1/50 dilution of the primary antibody (CK1α from Santa Cruz Biotechnology) was used to incubate the coverslips for 1 h. After washing with PBS, cells were incubated with the secondary antibody, Alexa Fluor 488 donkey anti-goat IgG (Invitrogen) diluted 1/400 in blocking solution, for 1 h at room temperature. Cells were washed again and incubated for 10 min with DAPI (4′,6-diamidino-2-phenylindole) for nucleus identification. Coverslips were mounted on glass slides with Mowiol, and immunofluorescence was viewed with a Leica confocal microscope (Leica spectral confocal TCS-SL).

CK1α activity assay. Proteins were immunoprecipitated from SW-480 total cell extracts (radioimmunoprecipitation assay [RIPA] buffer) (19) with CK1α MAb for 2 h at 4°C and then collected with 20 μl of γ-bind G-Sepharose (GE-Healthcare). Immunoprecipitates were washed three times with 25 mM Tris-HCl (pH 7.6)–0.1% NP-40, and the immunocomplexes were incubated with recombinant GST-p120-catenin under phosphorylation conditions. Phosphorylation assays were performed in the same conditions as before for 10 min at 30°C. Specific phosphorylation on GST-p120-catenin Ser268 was analyzed by Western blotting with a phospho-specific Ser268-p120-catenin MAb (25).

Luciferase reporter assays. HEK293 cells were transfected with TOP-Flash plasmid, a synthetic promoter sensitive to the activity of the β-catenin/Tcf-4 complex, that contains three copies of the Tcf-4 binding site upstream of a firefly luciferase reporter gene. A mutant form of this promoter (FOP plasmid) was used as a control. The activity of the product of the *Renilla* luciferase gene under the control of a constitutive thymidine kinase promoter (Promega) was used as to normalize transfection efficiency. Assays were performed in triplicate; the average of the results of three independent transfections (± standard deviation [SD]) is given.

RESULTS

CK1ε mediates transient CK1α interaction with p120-catenin. As mentioned above, CK1ε is constitutively bound to p120-catenin. This enzyme is activated by incubation with Wnt3a and is required for early responses to this factor, such as Dvl-2 phosphorylation (2; see also below). p120-catenin is also phosphorylated at Ser268 in response to Wnt3a, but this modification is barely observed before 30 min and is maximal at 1 to 2 h (Fig. 1A). Ser268 phosphorylation temporally correlates with the downregulation of the interaction of p120-catenin with E-cadherin and LRP5/6 (Fig. 1A).

Ser268 is a good substrate for a recombinant CK1 protein containing the catalytic fragment common to all of the CK1 isoforms (2). We examined if another isoform of this family is recruited to the p120-catenin family and contributes to Ser268 phosphorylation. Although not detected in unstimulated cells, CK1α immunoprecipitated with p120-catenin 20 and 30 min after Wnt3a addition (Fig. 1A, right panel). This association was transient since by 1 h it was downregulated, correlating with the breakdown of LRP5/6–p120-catenin interaction. We did not detect binding to p120-catenin of another CK1 isoform involved in Wnt signaling, CK1γ (Fig. 1A, bottom panel).

We determined if CK1α recruitment to LRP5/6 upon Wnt3a stimulation was dependent on CK1ε, as we have previously reported for p120-catenin Ser268 phosphorylation (see above). Specific depletion of CK1ε totally prevented the interaction of CK1α with p120-catenin observed after 30 min of incubation with Wnt3a (Fig. 1B, right panel, compare lanes 2 and 4). CK1α cellular levels were not modified by CK1ε shRNA. Wnt3a also induced the translocation of cellular CK1α that

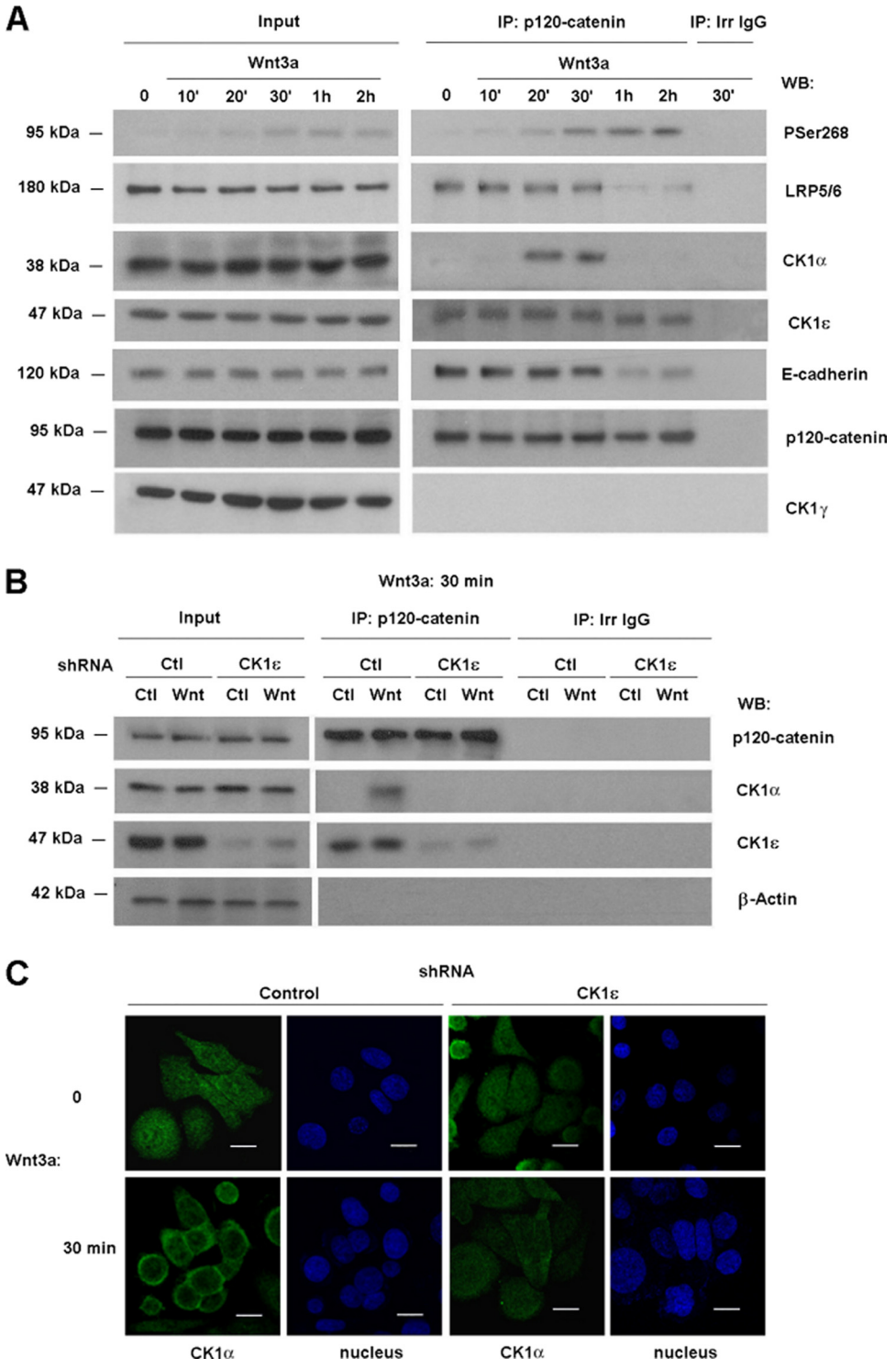

FIG. 1. CK1ε mediates transient CK1α interaction with p120-catenin upon Wnt3a stimulation. (A) SW-480 cells stimulated with Wnt3a-conditioned medium for the indicated times were lysed, and p120-catenin was immunoprecipitated (IP). Associated proteins were analyzed by Western blotting (WB). In the "input" lane, a sample corresponding to 5% of the lysates used was loaded. (B) SW-480 cells were transfected with scrambled shRNA or shRNA specific for CK1ε. Cells were treated with control (Ctl) or Wnt3a-conditioned medium for 30 min. p120-catenin was immunoprecipitated, and immunocomplexes were analyzed by Western blotting. (C) The subcellular distribution of CK1α was determined in SW-480 cells infected with scrambled shRNA or shRNA specific for CK1ε. Cells were treated with Wnt3a-conditioned medium for 0 min (upper panels) or 30 min (lower panels). The analysis was performed by immunofluorescence using an antibody against CK1α. No signal was obtained when the same analysis was performed in the absence of the primary antibody. The scale bar in each panel corresponds to 20 μm. All of the data presented in this figure are representative results from at least three different experiments.

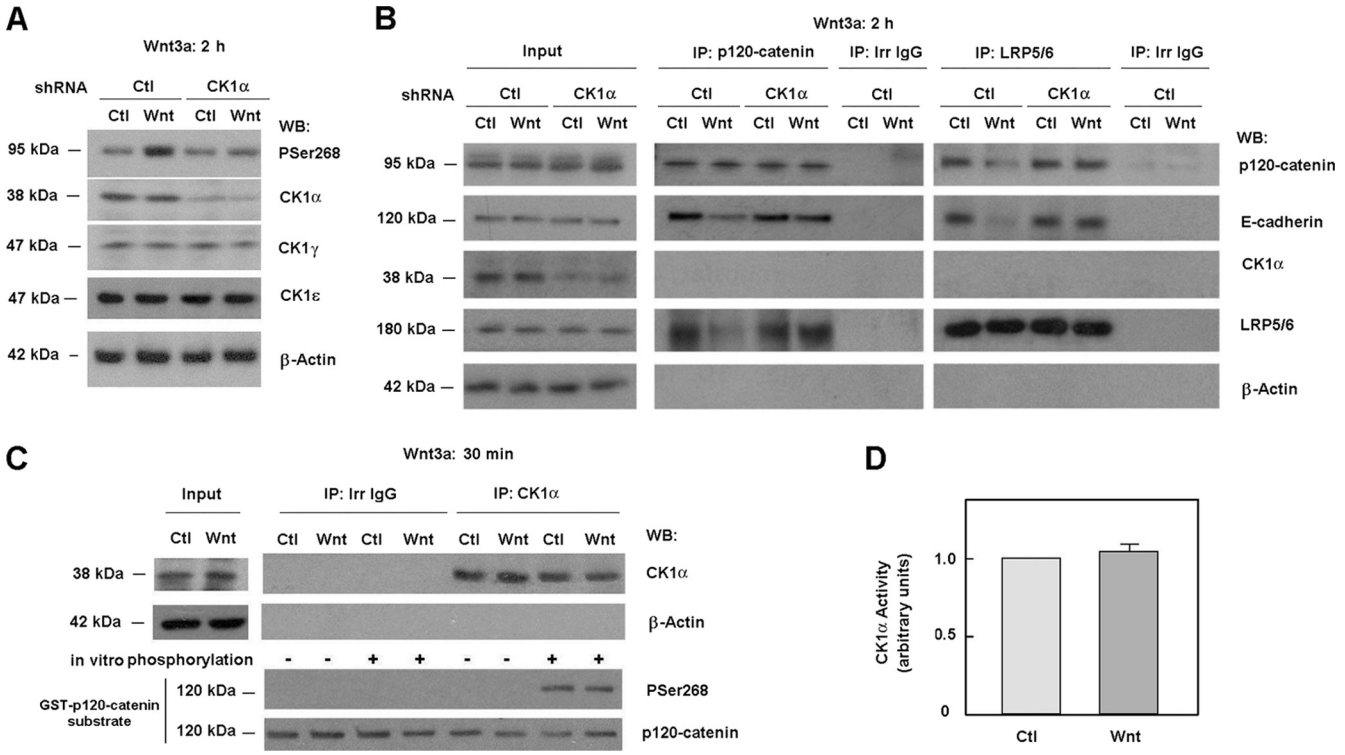

FIG. 2. CK1α is required for p120-catenin Ser268 phosphorylation and disruption of E-cadherin–p120-catenin interaction. (A) SW-480 cells were transfected with scrambled shRNA or an shRNA specific for CK1α and treated with control (Ctl) or Wnt3a-conditioned medium for 2 h. Cell extracts were analyzed by Western blotting (WB) with the indicated antibodies. (B) SW-480 cells depleted of CK1α as indicated above were stimulated or not with Wnt3a-conditioned medium for 2 h. Cells were lysed, and p120-catenin or LRP5/6 was immunoprecipitated (IP) with specific antibodies. Associated proteins were analyzed by Western blotting. In the "input" lane, a sample corresponding to 5% of each total cell extract was used. The results from a representative experiment out of three performed are shown. (C) SW-480 cells were treated with control or Wnt3a-conditioned medium for 30 min. Total cell extracts were immunoprecipitated with anti-CK1α antibody. Immunocomplexes were analyzed against CK1α or incubated with 2 pmol of recombinant GST-p120-catenin (positions 102 to 911) under CK1 phosphorylation conditions. Phosphorylation of Ser268 was analyzed by Western blotting with a specific phospho Ser268 antibody. The average ± standard deviation (SD) of the densitometric analysis of the results of the three experiments performed is presented in panel D. Phosphorylation of Ser268 was normalized considering the amount of substrate and referred to the value obtained in unstimulated cells. All of the data shown in this figure are representative of at least three independent experiments. Irr. IgG, an irrelevant IgG used as a control in the immunoprecipitation.

was mobilized to the cell membrane (Fig. 1C). As described above, depletion of CK1ε prevented this effect.

CK1α is required for p120-catenin Ser268 phosphorylation and disruption of E-cadherin/p120-catenin complex. The above-mentioned results indicate that the presence of CK1α in the LRP5/E-cadherin/p120-catenin complex temporally coincides with p120-catenin phosphorylation in Ser268. To examine the relevance of CK1α in this modification, a short hairpin RNA (shRNA) specific for CK1α and not affecting CK1ε or -γ (Fig. 2A) was used. Downregulation in the CK1α isoform prevented the increase in phospho Ser268 observed 2 h after Wnt3a stimulation (Fig. 2A). Since this phosphorylation is required for p120-catenin release from its interaction with E-cadherin, elimination of CK1α kept the complex stable 2 h after Wnt3a induction, in contrast to what happened in control cells (Fig. 2B, middle panel). Thus, E-cadherin was detected in p120-catenin immunocomplexes after this time only in CK1α-depleted cells. Moreover, since E-cadherin bridges p120-catenin to the LRP5/6 receptor complex, CK1α depletion also affected the p120-catenin–LRP5/6 interaction that remained detectable after 2 h, in contrast to control cells (Fig. 2B). Therefore, CK1α was required for p120-catenin Ser268 phosphorylation and the disruption of p120-catenin–E-cadherin binding.

We also determined if CK1α was capable of *in vitro* phosphorylating p120-catenin. CK1α was immunoprecipitated, and the protein kinase activity of the complex was assayed using GST-p120-catenin as a substrate (Fig. 2C). Ser268 phosphorylation was detected when recombinant p120-catenin was phosphorylated with a CK1α immunocomplex and not with the complex obtained with an irrelevant IgG (Fig. 2C, lower panel). No significant differences were observed between the activity of a CK1α immunocomplex obtained from control or Wnt3a-treated cells (Fig. 2C, lower panel, lanes 7 and 8, and Fig. 2D). This result confirms that CK1α is also a p120-catenin kinase and suggests that Wnt3a modulates CK1α recruitment to this substrate and not its intrinsic activity.

CK1ε but not CK1α is required for very early responses to Wnt3a. We compared the effects of the interference in CK1ε and -α in the molecular responses to Wnt3a. A time course analysis indicated that the upregulation in the phosphorylation of Dvl-2 or Thr1479 in LRP5/6 preceded that of p120-catenin Ser268 (Fig. 3A and B). The effects of the interference of the two CK1 isoforms were different: whereas depletion of CK1ε

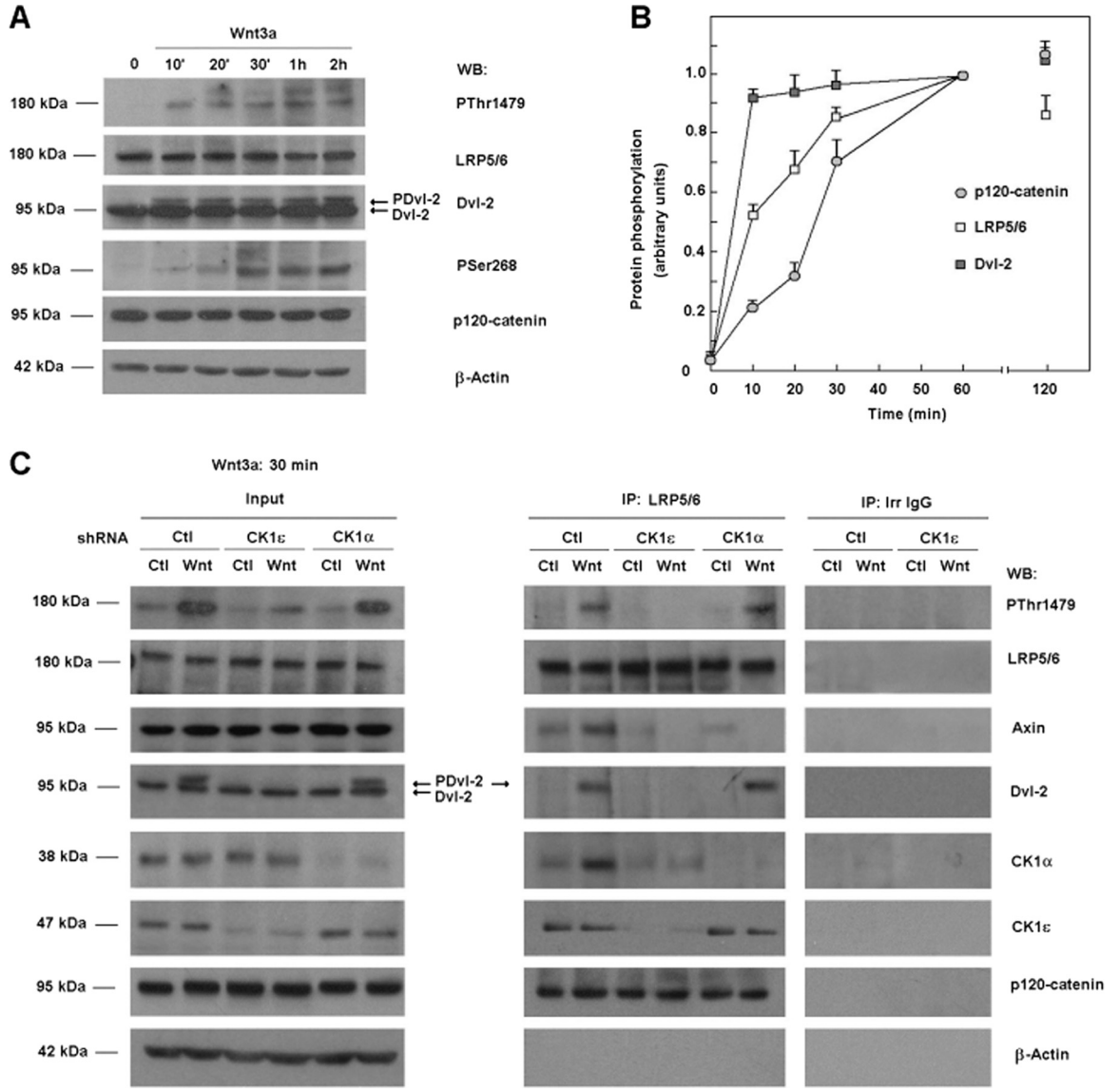

FIG. 3. CK1ε but not CK1α is required for very early events in Wnt3a signaling. (A and B) SW-480 cells were incubated with control (Ctl) or Wnt3a-conditioned medium for the indicated times, and total cell extracts were analyzed by Western blotting (WB) with the indicated antibodies. Representative blots are presented in panel A; shown is the average ± SD of the results of the three experiments performed in panel B. Protein phosphorylation was normalized considering the amount of each susbtrate and referred to the value obtained after 1 h of stimulation with Wnt. (C) SW-480 cells were transfected with scrambled or CK1ε- or CK1α-specific shRNAs. After selection with 2 μg/ml puromycin, cells were treated with control or Wnt3a-conditioned medium for 30 min. LRP5/6 was immunoprecipitated (IP) from total cell extracts, and associated proteins were analyzed by Western blotting. A representative Western blot of four different experiments is shown.

prevented the initial responses to Wnt3a (Dvl-2 and LRP5/6 Thr1479 phosphorylation), that of CK1α did not affect them (Fig. 3C, "input" lanes). We also examined the assembly of the signaling complex. CK1ε interference prevented the association of LRP5/6 with Dvl-2 that occurs preferentially with the phosphorylated form upon Wnt3a stimulation (Fig. 3C, right panel, lanes 2 and 4). Since Dvl-2 binding is required for the further interaction with axin, this protein did not associate with the complex in CK1ε-depleted cells. On the contrary, CK1α downregulation did not modify LRP–Dvl-2 binding but blocked the formation of the LRP/axin complex (Fig. 3C, right panel). As shown before, whereas binding of CK1ε to LRP5/6 was not affected by CK1α depletion, that of CK1ε inhibited CK1α recruitment to the LRP5/6 complex. Therefore, these results indicate that CK1α effects occur later than CK1ε effects, modifying either axin or LRP5/6 and preventing axin recruitment.

Axin binds and recruits CK1α to the LRP5/6 receptor complex. The kinetics of axin binding to LRP5/6 correlated with that of CK1α (Fig. 4A, right panel). Both CK1α and axin were detected in LRP5/6 complexes after 30 min of incubation with Wnt3a and were downregulated at later times. In contrast, CK1ε was constitutively bound and started to diminish by 30 min, identically to p120-catenin or E-cadherin (Fig. 4A). CK1α

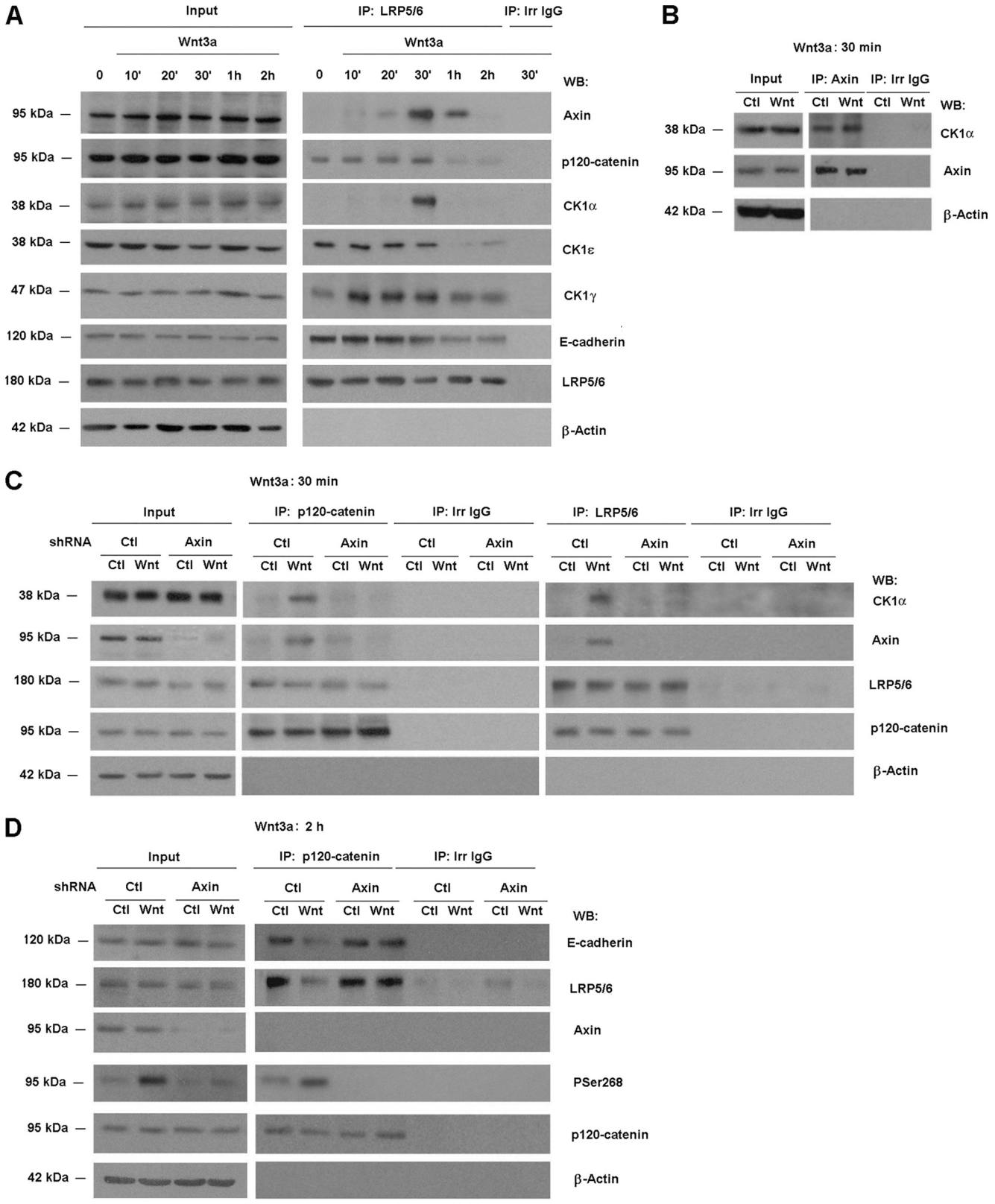

FIG. 4. Axin binds and recruits CK1α to the LRP5/6 receptor. (A) SW-480 cells stimulated or not with Wnt3a-conditioned medium for the indicated times were lysed, and LRP5/6 was immunoprecipitated (IP). Associated proteins were analyzed by Western blotting (WB) with the indicated antibodies. In the "input" lane, a sample corresponding to 5% of the lysates used was loaded. (B) SW-480 cells were treated with control (Ctl) or Wnt3a-conditioned medium for 30 min. Axin was immunoprecipitated, and immunocomplexes were analyzed against CK1α by Western blotting. (C and D) SW-480 cells were depleted of axin by using a specific shRNA. After selection with 2 μg/ml puromycin, cells were stimulated with control or Wnt3a-conditioned medium for 30 min (C) or 2 h (D). p120-catenin (C and D) or LRP5/6 (C) was immunoprecipitated from whole-cell extracts, and the associated proteins were analyzed by Western blotting. β-Actin was used as a negative control. All of the data presented in this figure are representative of at least three independent experiments.

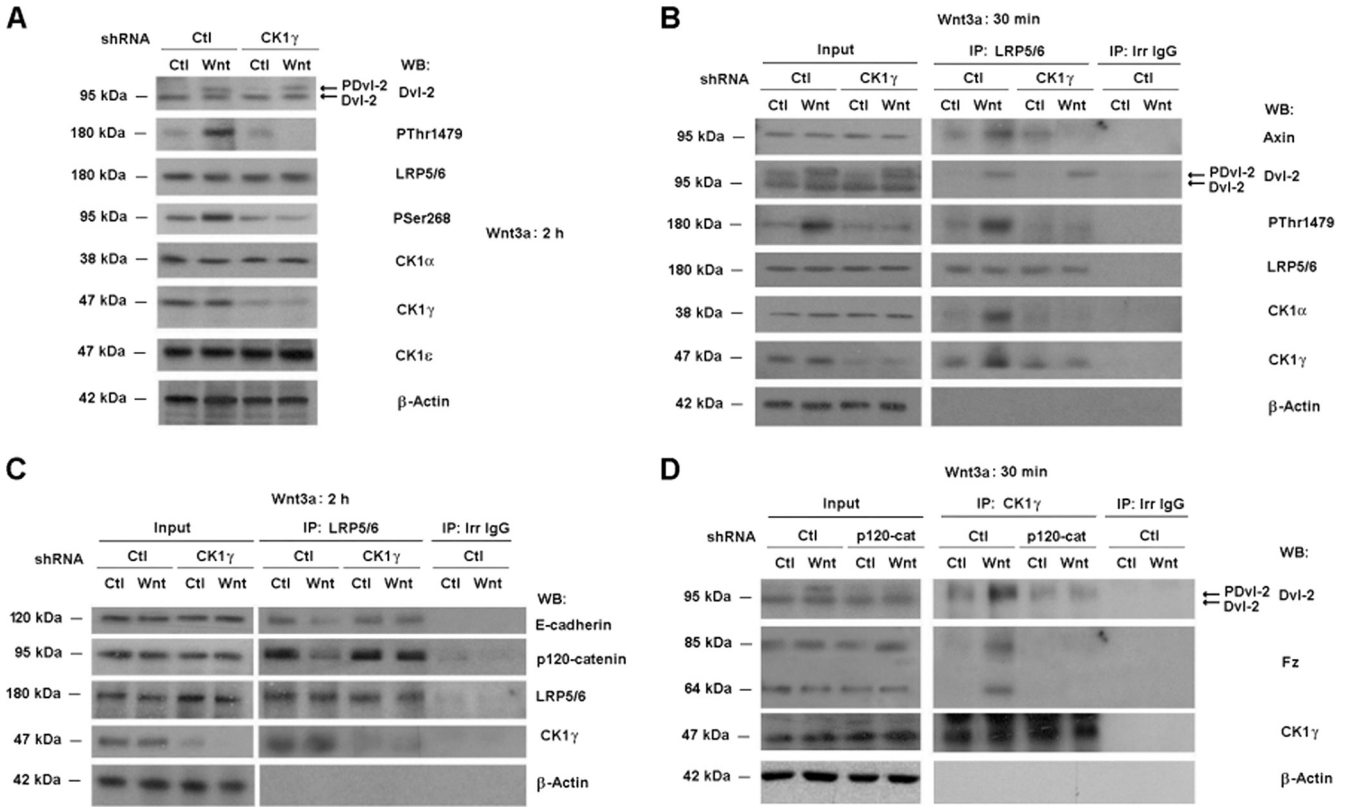

FIG. 5. CK1γ is required for Wnt3a-induced LRP5/6 T1479 phosphorylation but not for Dvl-2 binding to LRP5/6. SW-480 cells were depleted of CK1γ or p120-catenin by using specific shRNAs or a scrambled shRNA as a control (Ctl). After the selection, cells were treated with control or Wnt3a-conditioned medium for 30 min (B and D) or 2 h (A and C), respectively. (A) Total cell extracts were analyzed by Western blotting (WB) using antibodies against the indicated proteins. (B, C, and D) Cell extracts were immunoprecipitated (IP) with anti-LRP5/6 (B and C) or CK1γ (D), and associated proteins were analyzed by Western blotting with the indicated antibodies. The results from a representative experiment out of three performed are shown.

and axin interact, since CK1α was detected in axin immunocomplexes independently of Wnt3a (Fig. 4B). In order to analyze if axin was the responsible for anchoring CK1α to the signaling complex, axin levels were downregulated with a specific shRNA. Axin depletion did not affect the integrity of the LRP5/p120-catenin complex upon 30 min of Wnt3a addition (Fig. 4C); however, it prevented the interaction of CK1α with p120-catenin. Similarly, the association of CK1α with LRP5/6 was also blocked by axin shRNA (Fig. 4C).

Later responses to Wnt3a (2 h) were also determined. As expected, since it disrupts CK1α interaction with the complex, axin depletion prevented Wnt3a effects dependent on this kinase—thus, p120-catenin Ser268 phosphorylation and the disruption of p120-catenin association with E-cadherin and LRP5/6 (Fig. 4D).

CK1γ depletion affects LRP5/6 Thr1479 phosphorylation but not Dvl-2 binding to LRP5/6. Our results indicate that the two CK1 isoforms play a positive but distinct role in Wnt signaling, with CK1ε being involved in the very early responses (such as Dvl-2 binding to the complex), whereas CK1α acts later and is recruited with axin to the receptor complex. We compared these two functions with that of a third member of this family, CK1γ, also involved in Wnt signaling. We followed a similar experimental approach downregulating this isoform with a specific shRNA that did not affect CK1ε or CK1α protein levels (Fig. 5A). CK1γ depletion prevented Wnt3a-induced LRP5/6 phosphorylation in Thr1479, but did not affect Dvl-2 phosphorylation (Fig. 5A).

Coimmunoprecipitation experiments were consistent with these results. Therefore, 30 min after addition of Wnt3a, CK1γ depletion inhibited axin recruitment to LRP5/6 (since it is dependent on Thr1479 phosphorylation) but not that of Dvl-2 (Fig. 5B). CK1α did not bind to LRP5/6 in CK1γ-depleted cells; consequently, p120-catenin was not phosphorylated in Ser268 (Fig. 5A), and p120-catenin and E-cadherin remained associated with LRP5/6 after 2 h of exposure to Wnt3a, in contrast to control cells in which the complex was disrupted (Fig. 5C). Therefore, these results indicate that CK1γ acts earlier in the Wnt3a pathway than CK1α but later than CK1ε.

We also analyzed if CK1γ is constitutively associated with some of the Wnt signalosome components. As shown in Fig. 5B, binding of this protein kinase to LRP5/6 was enhanced after 30 min of Wnt3a addition. Similar results were obtained when the interaction with Fz or Dvl-2 was examined (Fig. 5D). The association was detected with phosphorylated Dvl-2, further stressing the importance of CK1ε, essential for this translational modification (Fig. 3C), in the association of CK1γ with the complex.

CK1ε is also required for CK1α association with LRP5/6 in HEK293 cells. We verified whether this successive action of

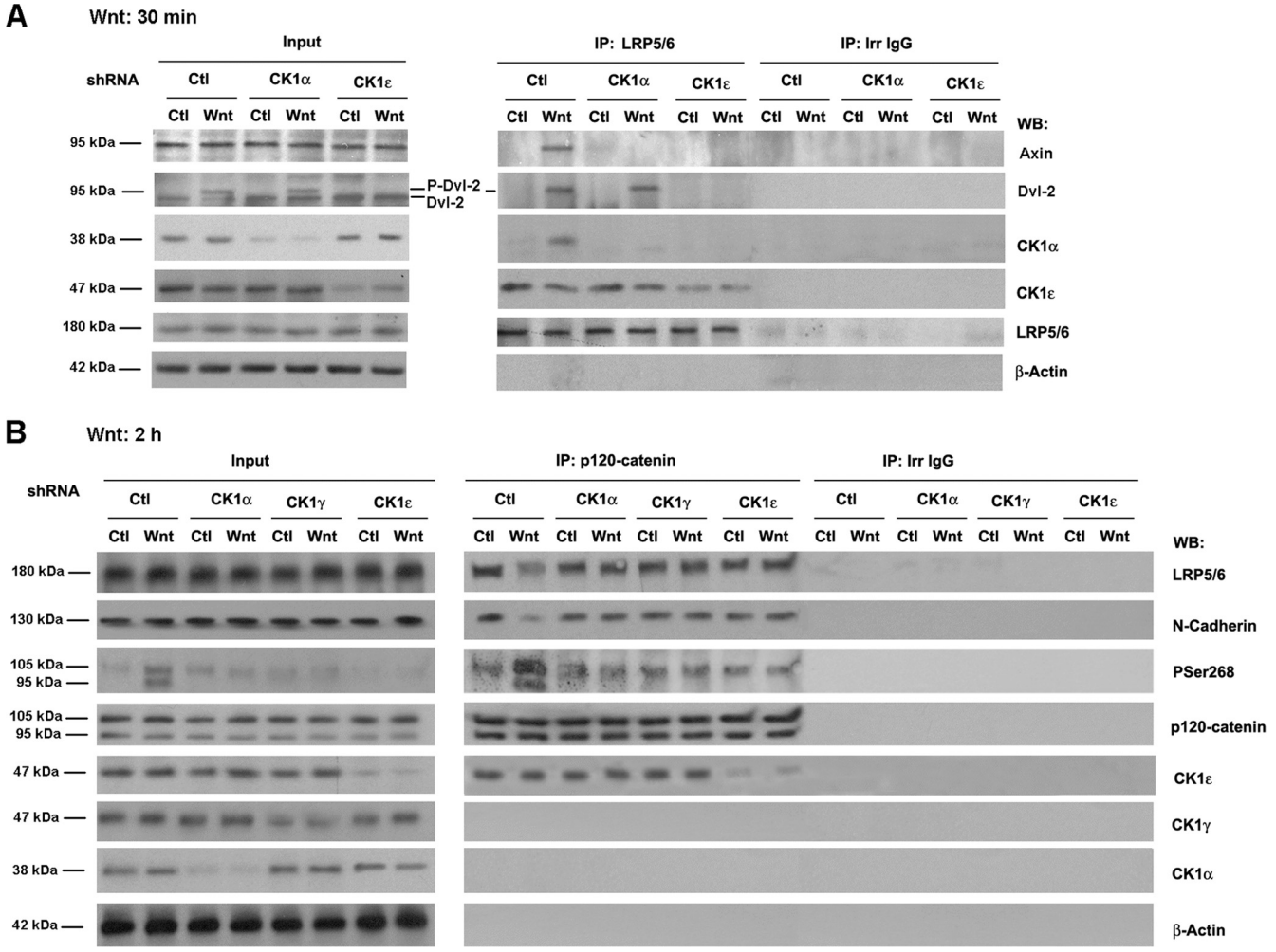

FIG. 6. CK1ε is required for CK1α interaction with LRP5/6 in HEK293 cells. HEK293 cells were transfected with scrambled or CK1α- or CK1ε-specific shRNA for 72 h (A) or scrambled, CK1α- CK1ε-, or CK1γ-specific shRNA for 72 h (B). Cells were treated with control (Ctl) or Wnt3a-conditioned medium for 30 min (A) or 2 h (B). LRP5/6 (A) or p120-catenin (B) was immunoprecipitated (IP) from total cell extracts, and immunocomplexes were analyzed by Western blotting (WB). In the "input" lane, a sample corresponding to 5% of the lysates used was loaded. All of the data presented in this figure are representative of at least three independent experiments. Irr. IgG, an irrelevant IgG used as a control in the immunoprecipitation.

CK1 isoforms in Wnt signaling is also observed in other cell lines. HEK293 cells have been broadly used to study Wnt effects. We downregulated CK1ε or CK1α expression in these cells using specific shRNAs. As shown in Fig. 6, depletion of both isoforms differently affected Wnt3a responses. CK1ε-depleted cells did not show upregulated Dvl-2 phosphorylation 30 min after Wnt3a stimulation (Fig. 6A, "input" lanes). Dvl-2 association with LRP5/6 was also inhibited as well as the recruitment of axin or CK1α to the LRP5/6 complex (Fig. 6A). On the contrary, CK1α interference did not modify Dvl-2 phosphorylation or its interaction with LRP5/6 and only affected axin binding to the coreceptor, as previously shown in SW-480 cells.

Later effects, such as p120-catenin phosphorylation in Ser268 and release of p120-catenin from LRP5/6 were affected by depletion of CK1α, -ε, or -γ isoforms (Fig. 6B). As shown in this figure, HEK293 cells express p120-catenin isoforms 1 and 3, with molecular masses of 105 kDa and 95 kDa, respectively; both isoforms contain Ser268 and were phosphorylated 2 h after Wnt3a addition. Depletion of the three CK1 isoforms prevented the upregulation of phospho Ser268, as well as the downregulation in the amount of p120-catenin coimmunoprecipitated with LRP5/6 or with N-cadherin, the most abundant cadherin in these cells. These results indicate that also in these cells CK1ε is required for CK1α binding to the complex, but the latter enzyme is responsible for p120-catenin phosphorylation and disruption of its interaction with N-cadherin.

Similarly to what we have described in SW-480 cells, in HEK293 cells CK1ε is constitutively bound to LRP5/6 (Fig. 6A). We have previously described that the interaction of p120-catenin/CK1ε complex with LRP5/6 is mediated by E-cadherin (2). However, HEK293 cells lack E-cadherin, although they express N-cadherin. Therefore, we determined whether N-cadherin also interacted with LRP5/6. As previously described by other authors (6), LRP5/6 interacted with N-cadherin since it was efficiently coimmunoprecipitated by

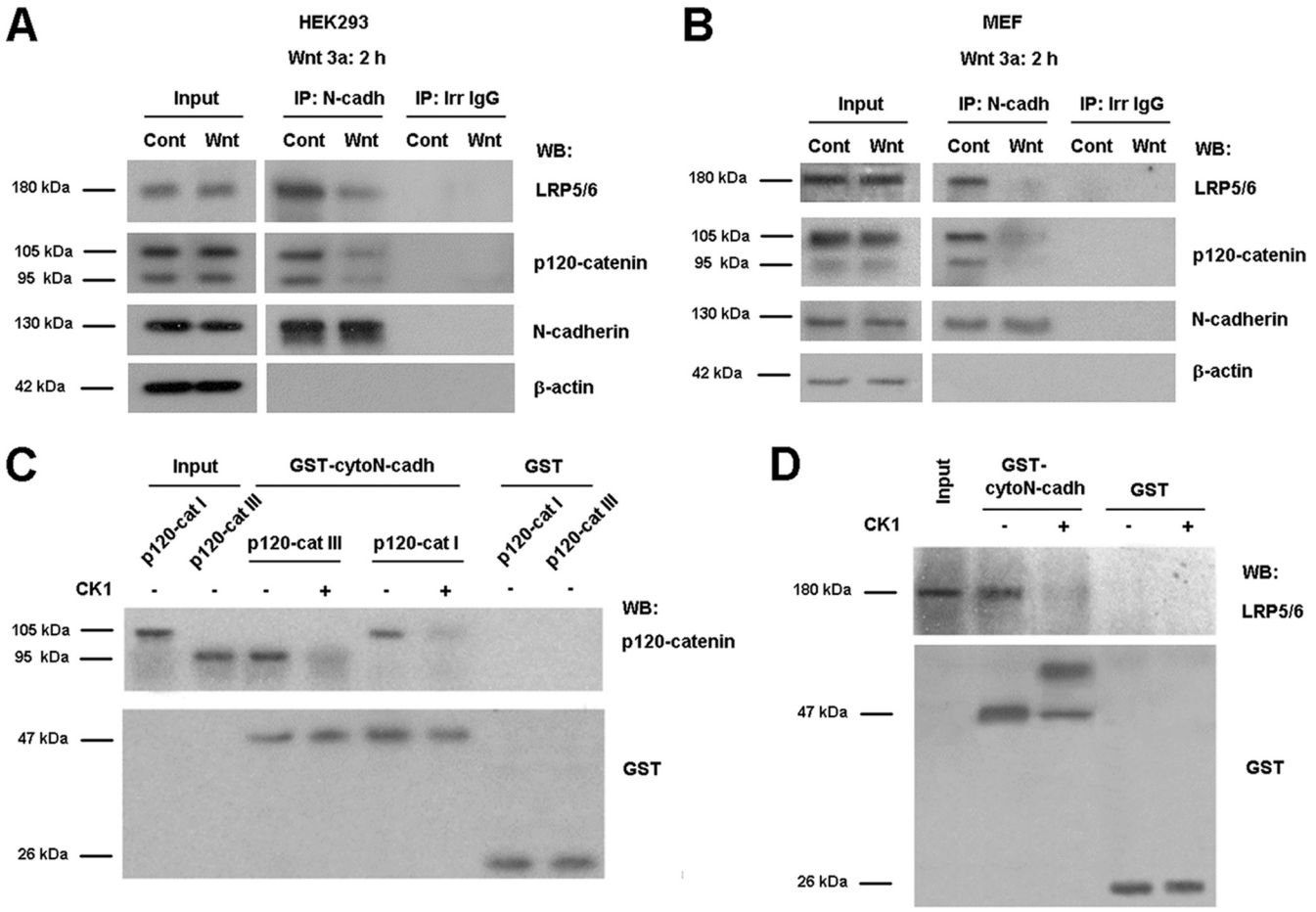

FIG. 7. N-cadherin–LRP5/6 interaction is sensitive to Wnt3a and CK1 phosphorylation. HEK293 cells (A) or MEF cells (B) were treated with control or Wnt3a-conditioned medium for 2 h. N-cadherin was immunoprecipitated (IP) from total cell extracts, and the presence in the immunocomplex of p120-catenin and LRP5/6 was determined by Western blotting (WB). In the "input" lane, 5% of each total cell extract used was loaded. (C) Recombinant p120-catenin corresponding to isoform 1 (aa 1 to 911) or 3 (aa 102 to 911) was *in vitro* phosphorylated with the CK1 catalytic domain when indicated. *In vitro* binding assays were performed by incubating recombinant GST-cytoN-cadh (3 pmol) or GST as a control with p120-catenin (aa 1 to 911) (2 pmol) or p120-catenin (aa 102 to 911). Protein complexes were affinity purified with glutathione-Sepharose and analyzed by Western blotting with anti-p120-catenin. Blots were reanalyzed with anti-GST to ensure that a similar amount of fusion protein was present. (D) Recombinant GST fusion proteins containing cytoN-cadh or GST as a control were *in vitro* phosphorylated with the CK1 kinase domain when indicated. Pulldown assays were performed by incubating fusion proteins (7 pmol) with extracts from 293T cells. Protein complexes were affinity purified and analyzed by Western blotting.

N-cadherin-specific antibodies in 293 cells (Fig. 7A) and MEFs (Fig. 7B). LRP5/6–N-cadherin binding was decreased after 2 h of incubation with Wnt3a, similar to what was previously shown for LRP5/6–E-cadherin association (Fig. 2B).

The interaction of N-cadherin with LRP5/6 and p120-catenin was also verified by pulldown assays. Two p120-catenin recombinant proteins encompassing aa 1 to 911 and 102 to 911 (corresponding to isoforms 1 and 3, respectively) were efficiently retained by a GST fusion protein containing the cytosolic domain of N-cadherin (cytoN-cadh) (Fig. 7C). The binding was sensitive to p120-catenin phosphorylation, since incubation of this protein with the recombinant catalytic fragment of CK1 prevented the interaction with N-cadherin. LRP5/6 was also retained by a GST-cytoN-cadh (Fig. 7D). Phosphorylation of this N-cadherin fragment with recombinant CK1 induced a shift in the molecular weight of N-cadherin, indicating extensive phosphorylation, and totally prevented LRP5/6 binding (Fig. 7D). These results totally mimic those previously reported by us concerning LRP5/6 interaction with E-cadherin (2) and demonstrate that both cadherin proteins behave identically with respect to their interaction with p120-catenin and LRP5/6.

Finally, we investigated the relevance of the different CK1 isoforms in the final responses of Wnt signaling: β-catenin stabilization and stimulation of β-catenin transcriptional activity. Depletion of both CK1ε and CK1γ isoforms did not significantly affect the basal levels of β-catenin (Fig. 8A); cells knocked down in these isoforms did not respond to Wnt3a with β-catenin accumulation. CK1α downregulation increased β-catenin basal levels in accordance with the role of this isoform in β-catenin phosphorylation and degradation (see the introduction); these cells did not respond either to Wnt3a with a further β-catenin upregulation.

β-Catenin/Tcf-4 transcriptional activity was also measured

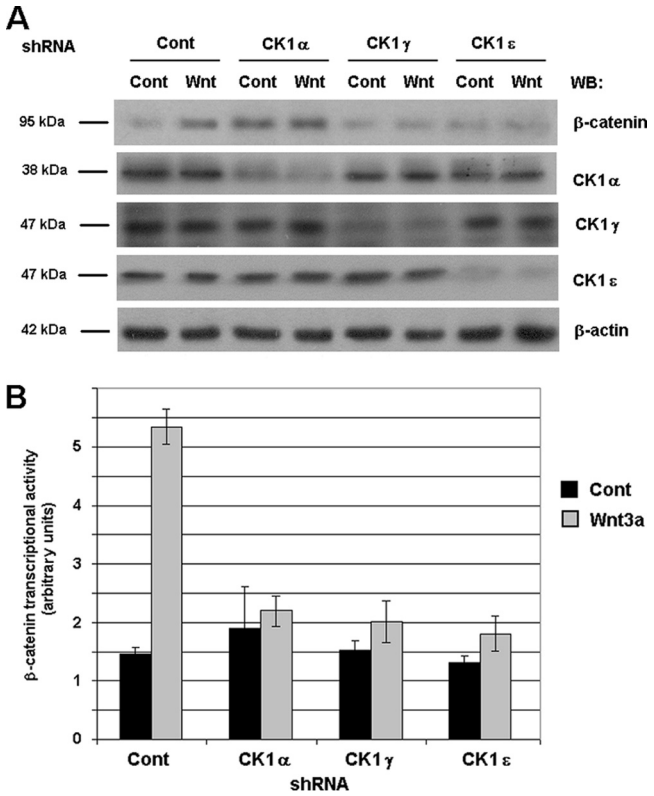

FIG. 8. CK1ε, -γ, and -α isoforms are required for Wnt3a stimulation of β-catenin/Tcf-4 transcriptional activity. (A) HEK293 cells were depleted of CK1α, CK1ε, or CK1γ by using specific shRNAs and treated with control (Cont) or Wnt3a-conditioned medium for 16 h. Cells were lysed in RIPA buffer to obtain total extracts. Levels of total β-catenin were determined by Western blotting (WB). The results from a representative experiment out of three performed are shown. (B) β-Catenin transcriptional activity was determined using the TOP reporter plasmid in HEK293 cells transfected with scrambled, CK1α, CK1ε, or CK1γ shRNA. pTK-*Renilla* plasmid was transfected to normalize the efficiency of transfection. Relative luciferase activity was determined 48 after transfection, when the indicated cells were treated for the last 16 h with Wnt3a-conditioned medium. The results show the average ± range of two experiments performed in quadruplicate.

with the TOP reporter plasmid. Contrary to control cells, Wnt3a only stimulated very slightly TOP activity in cells depleted of each of the three isoforms (Fig. 7B). It is interesting to remark that although CK1α depletion upregulated β-catenin protein, it did not increase β-catenin transcriptional activity, suggesting that CK1α also plays a positive role in the Wnt3a pathway.

DISCUSSION

Different protein kinases are involved in Wnt signaling. Upon binding of canonical Wnt ligands to high-affinity Frizzled (Fz) receptors, the coreceptor LRP5/6 is recruited and a ternary complex, Fz/Wnt/LRP5, is formed. Other proteins, such as Dvl-2 and axin, are successively coupled, originating a high-molecular-weight receptor-ligand complex that has been named "Wnt signalosome" (1). In this complex, LRP5/6 is phosphorylated by several kinases, which enables binding of GSK-3β, the enzyme responsible for targeting β-catenin for destruction by the proteasome. Binding of GSK-3β inhibits this kinase either by facilitating its internalization in multivesicular vesicles (22) or by creating an inhibitory site (24). Besides GSK-3β itself, the CK1ε, -α, and -γ isoforms and Grk5/6 phosphorylate different sites in LRR5/6 (15). However, determination of the precise CK1 family member involved in the modification of a specific residue has been hindered by the very similar specificity of these kinases, which share an almost common catalytic domain (10). Therefore, *in vitro* most of these kinases are capable of phosphorylating substrates modified *in vivo* by another member of the family. In this work, we have described how the different CK1 isoforms are sequentially bound to the receptor and are required for different steps in the formation of the Wnt signalosome.

As we show here and have previously reported (2), LRP5/6 is constitutively bound to CK1ε. This association is not direct, but it is mediated by E-cadherin and p120-catenin (as modeled in Fig. 9A). E-cadherin function can also be conducted by N-cadherin in mesenchymal cells or probably by other cadherins since the cytosolic domain of all these proteins is very homologous. Differently from other members of the CK1 family, CK1ε can be detected in both active and inactive states, dependent on the phosphorylation of the C-tail, which creates a self-inhibitory site (10). In unstimulated cells, p120-catenin-bound CK1ε is inactive. Upon Wnt3a stimulation, the complex containing LRP5/6, E-cadherin, p120-catenin, and CK1ε rapidly associates with Fz, concomitantly with the activation of this protein kinase (Fig. 9B). It is possible that a still not characterized protein phosphatase is recruited by Fz, enabling its action on the LRP5-associated CK1ε and the activation of this enzyme.

Both CK1ε and p120-catenin are required for Dvl-2 phosphorylation and binding to Fz/LRP5/6 complex. Dvl-2 is a good CK1 substrate *in vitro*. It is possible that Dvl-2 phosphorylation by CK1ε increases its weak intrinsic affinity for Fz (23), stabilizing the association of Dvl-2 with the Wnt receptor complex (Fig. 9C). Alternatively, CK1ε might modify LRP5/6 and create a new Dvl-2 binding site that contributes to strengthen this association.

Besides, Dvl-2 phosphorylation stimulates the interaction of axin with the complex, probably acting at two levels. First, Dvl-2 is required for binding of CK1γ to the complex (28), which enables the phosphorylation of LRP5/6 at Thr1479, which is critical for axin recruitment. Binding of CK1γ is probably due to an increased affinity for this protein caused by phosphorylation of Dvl-2; according to this hypothesis, phosphorylated Dvl-2 would act as the CK1γ-docking element to the receptor complex. Moreover, phosphorylated Dvl-2 polymerizes, which is necessary for the formation of the signalosome and likely also increases the avidity for axin.

In any case, as the result of CK1γ-dependent Thr1479 phosphorylation, axin binds to LRP5/6 (Fig. 9D). Axin is constitutively associated with active CK1α and is required for the interaction of this protein with the complex. Our results indicate that CK1α is also required for the association of axin with LRP5/6 (Fig. 3C). Since axin is a phosphoprotein (26), it is likely that CK1α also modifies this protein, contributing to increase its affinity for phosphorylated LRP5/6. In addition to CK1α, axin likely binds to GSK-3β and enables its interaction with LRP5/6, in a similar fashion to its role in the APC-

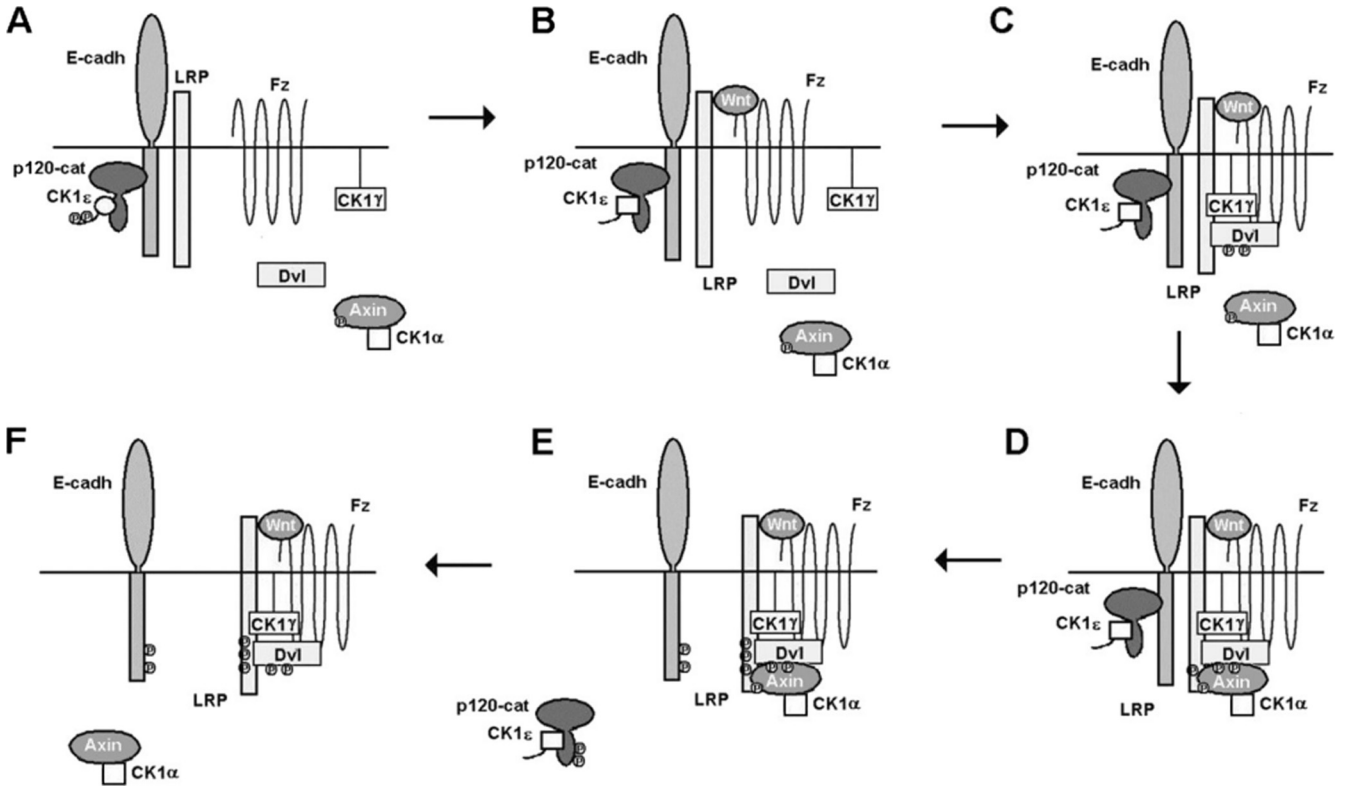

FIG. 9. Proposed model for the involvement of the different CK1 isoforms in the Wnt pathway. In unstimulated cells (A), inactive CK1ε is bound to p120-catenin and E-cadherin (E-cadh); E-cadherin is also associated with Wnt coreceptor LRP5/6 (LRP). CK1α is interacting with axin. Upon binding of Wnt ligands and the formation of the LRP5/6-Fz complex (B), CK1ε is activated by removal of inhibitory phosphates in its C-terminal tail by the action of an unknown phosphatase. Activated CK1ε phosphorylates Dvl-2 (Dvl), stabilizing the interaction of Dvl-2 with LRP5/6 (C). Binding of Dvl-2 enables LRP5/6 phosphorylation at Thr1479 by the action of CK1γ, which in turn allows the recruitment of axin to the complex (D). Axin-associated CK1α also binds to the Wnt-receptor complex, contributing to LRP5/6 phosphorylation (E). Moreover, CK1α also phosphorylates p120-catenin at Ser268 and E-cadherin, releasing E-cadherin and p120-catenin from the signalosome. CK1ε is released from the complex with p120-catenin, interrupting the input signal. Finally, axin/CK1α complex is released from LRP5/6-Dvl2 complex by the action of a phosphatase that dephosphorylates axin (F).

dependent β-catenin degradation complex (13). In any case, the association of CK1α and maybe GSK3-β causes a more extensive phosphorylation of LRP5/6.

Furthermore, CK1α phosphorylates E-cadherin in a residue yet to be identified and p120-catenin at serines 268 and 269 (Fig. 9E). These modifications disrupt the interaction of E-cadherin with LRP5/6 and that of p120-catenin with E-cadherin, respectively (2), and release p120-catenin-bound CK1ε from the signaling complex (Fig. 9E). The release of p120-catenin/CK1ε has two different consequences: first, it enables p120-catenin to perform further actions on this pathway, such as binding to Kaiso and modulation of the negative action of this factor on β-catenin/Tcf-4 transcriptional activity (5). This action of p120-catenin in the Wnt pathway, dependent on CK1α, might explain why CK1α-deficient cells do not show an upregulated β-catenin-dependent transcription, although they present stabilized β-catenin. Besides being relevant for p120-catenin–Kaiso binding, removal of p120-catenin from the LRP5/6 complex promotes the cessation of the initial input since it uncouples CK1ε from the signalosome, preventing further Dvl-2 phosphorylation. Therefore, the disruption of the complex will temporally limit the cellular response to canonical Wnt.

Finally, axin/CK1α is released from the LRP5/6 complex. We have detected that the axin-LRP interaction is transient: it is detected at 30 min but not at 2 h of Wnt3a incubation (Fig. 4A). It is possible that axin release is dependent on a phosphatase acting on this protein that dephosphorylates residues critical for the association with LRP5/6. In this respect, that protein phosphatase 1 can interact with and dephosphorylate axin has been described (8). Axin removal will also contribute to the elimination of the input signals that activate this pathway.

Downregulation of the receptor in response to a sustained activation is a general phenomenon (see, for instance, reference 20 for the β-adrenergic receptors) that frequently involves protein kinases that inactivate the receptor complex. In this case, we propose that CK1α is the protein kinase playing this role, since it is the enzyme responsible for the disruption of the interaction among p120-catenin and E-cadherin with LRP5/6. Moreover, and considering the close similarity between the catalytic domains of CK1ε and CK1γ, it is possible that CK1α is indeed responsible for the phosphorylation at Ser1420 and Ser1430 in LRP5/6 that negatively affects Wnt signaling by still not defined mechanisms (21). Another idea coming from our results is that the extent of Wnt signalosome

downregulation might be dependent on the cellular levels of axin, since this protein recruits CK1α to the complex. This protein has been reported to be present at low levels in most cells (11).

In summary, we propose a mechanism, consistent with the results published here and in many other articles, that explains the activation of the Wnt signalosome. Although some issues are not detailed here (for instance, whether other p120-catenin family members behave as p120-catenin in this complex; what the role of other protein kinases, such as CK1δ or Grk5/6 is; and how axin's interaction with LRP5/6 is modulated by axin phosphorylation), we consider that this model based on the successive recruitment and action of CK1 family members can help us understand how the Wnt signaling pathway is activated.

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